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The endothelial cell protein C receptor augments protein C activation by the thrombin–thrombomodulin complex

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ABSTRACT **Protein C activation on the surface of the endothelium is critical to the negative regulation of blood coagulation. We now demonstrate that monoclonal antibodies that block protein C binding to the endothelial cell protein C receptor (EPCR) reduce protein C activation rates by the thrombin–thrombomodulin complex on endothelium, but that antibodies that bind to EPCR without blocking protein C binding have no effect. The kinetic result of blocking the EPCR–protein C interaction is an increased apparent K_m for the activation without altering the affinity of thrombin for thrombomodulin. Activation rates of the protein C derivative lacking the γ-carboxyglutamic acid domain, which is required for binding to EPCR, are not altered by the anti-EPCR antibodies. These data indicate that the protein C activation complex involves protein C, thrombin, thrombomodulin, and EPCR. These observations open new questions about the control of coagulation reactions on vascular endothelium.**

Our current concepts of the mechanisms of the protein C anticoagulant pathway are that the pathway is triggered when thrombin binds to the endothelial cell receptor, thrombomodulin (TM) (reviewed in refs. 1–4). This complex activates protein C to generate the anticoagulant enzyme activated protein C (APC), which, in complex with protein S, inhibits coagulation by inactivating two critical regulatory proteins, factors Va and VIIIa. This pathway plays a critical role in the negative regulation of blood coagulation, as evidenced by the fact that total deficiencies of protein C or protein S are associated with severe and life-threatening thrombotic complications (reviewed in ref. 2), and deletion of the TM gene in mice results in embryonic lethality (5).

We recently identified and cloned the endothelial cell protein C/APC receptor (EPCR) (6). EPCR is a type 1 transmembrane protein that binds both its ligands with similar affinity ($K_d \approx 30$ nM) (7) and inhibits the ability of APC to inactivate its natural substrate, factor Va (8). Like TM, EPCR is down-regulated by tumor necrosis factor α on endothelium *in vitro*, and the down-regulation of the two receptors follows the same time course, suggesting that these two receptors might function in a common pathway (6).

Several of the requirements for rapid protein C activation by the endothelium overlap the requirements for ligand binding to EPCR. In particular, proteolytic removal of the vitamin K-dependent γ-carboxyglutamic acid (Gla) domain of protein C prevents binding to EPCR and reduces the activation rate of protein C over endothelium at least 5-fold (9). In contrast, purified TM accelerates protein C activation to the same extent in solution irrespective of the presence of the protein C Gla domain (9). Furthermore, a subgroup of rabbit endothelial cell lines were identified in earlier studies that, unlike primary human or bovine endothelium, failed to distinguish between protein C and the derivative devoid of the protein C Gla domain (10, 11). Taken together, these data supported the possibility that an additional endothelial cell protein might facilitate protein C activation.

In this study, we describe monoclonal antibodies (mAbs) that bind to EPCR and block both protein C and APC binding to cells stably transfected with EPCR (E7 cells), to human umbilical endothelial cells (HUVECs), and to EA.hy926 cells (a transformed endothelial cell line). On those cells that express both TM and EPCR, these anti-EPCR antibodies inhibit thrombin-dependent protein C activation, but not the activation of Gla-domainless protein C. This is, to our knowledge, the first evidence that EPCR participates in the process of protein C activation and the natural anticoagulant pathway.

EXPERIMENTAL PROCEDURES

Reagents. Suppliers were as follows: fluorescein thiosemicarbazide, Molecular Probes; biotinamidocaproate *N*-hydroxysuccinimide ester, avidin, and bovine serum albumin (BSA), Sigma; streptavidin-phycoerythrin, Becton Dickinson; biotin-LC-hydrazide, Pierce; and Spectrozyme PCa, American Diagnostica. All other reagents were of the highest quality commercially available.

Protein Preparation. sEPCR-HPC4, the recombinant soluble form of human EPCR, was prepared as described previously (7). The construct codes for the extracellular domain of EPCR truncated immediately above the transmembrane domain at residue 210. An HPC4 epitope tag was attached to the carboxyl terminus for calcium-dependent affinity purification (12). Recombinant protein C lacking the Gla domain, Gla-domainless protein C, was prepared as described (12).

Bovine thrombin (13) and human protein C (14) and APC (14) were prepared as described. The extinction coefficients $[(mg/ml)^{-1} \cdot cm^{-1}]$ and molecular weights used for this study were as follows: bovine thrombin (2.1, 37,000) (15), human APC (1.45, 60,000) (13), human protein C (1.45, 62,000) (13), sEPCR-HPC4 (1, 42,000) (7), and Gla-domainless protein C (1.45, 58,000) (12).

The active site of APC was labeled with fluorescein to generate fl-APC as described (6). Protein C was directly labeled with fluorescein by using methods described to selec-

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Abbreviations: TM, thrombomodulin; APC, activated protein C; EPCR, endothelial protein C/APC receptor; sEPCR-HPC4, recombinant soluble human EPCR with the HPC4 epitope inserted in place of the transmembrane domain and cytosolic tail; fl-APC, APC with fluorescein attached to the active site; biotin-PC, protein C biotinylated on primary amino groups; fl-cho-protein C, protein C selectively labeled with fluorescein on sialic acids; biotin-cho-sEPCR-HPC4, soluble EPCR selectively labeled with biotin on sialic acids; Gla, γ-carboxyglutamic acid; FACS, fluorescence-activated cell sorter; mAb, monoclonal antibody; HUVECs, human umbilical vein endothelial cells.

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tively label terminal sialic acid groups (fl-cho-protein C) (16). Protein C (0.8 mg/ml in 0.1 M NaCl/0.1 M sodium acetate, pH 5.5) was incubated with 1 mM sodium metaperiodate for 20 min in the dark on ice. Sodium metabisulfite was added to 0.4 mM, the mixture was incubated for 5 min at room temperature, and fluorescein thiosemicarbazide was added to a final concentration of 0.5 mM. The solution was incubated for 60 min on ice in the dark and the fl-cho-protein C was dialyzed extensively at 4°C against Hanks balanced salt solution (HBSS). For some experiments, protein C (0.8 mg/ml at pH 8.5) was labeled with biotin on primary amino groups by incubation with biotinamidocaproate *N*-hydroxysuccinimide ester (final 0.2 mM) for 2 hr on ice. The reaction was quenched with 2 M Tris·HCl, pH 7.5, and the biotin-PC was dialyzed extensively against HBSS to remove free biotin.

Cell Culture. All human cell lines were maintained as described previously (7). E7 cells are human kidney 293 cells stably transfected with full-length human EPCR and have been characterized previously (7). HUVECs were kindly provided by Craig Carson (Oklahoma City Clinic). EA.hy926 cells, a transformed human endothelial cell line (17), were kindly provided by Cora-Jean Edgell (University of North Carolina at Chapel Hill).

mAbs and Screening. mAbs against sEPCR-HPC4 were obtained as described previously for other proteins (14). The hybridoma supernatants were screened by fluorescence-activated cell sorter (FACS) analysis, using a FACSCalibur flow cytometer (Becton Dickinson), for their ability to block the binding of 80 nM fl-APC or labeled protein C to E7 and EA.hy926 cells. Cells were incubated with hybridoma supernatants containing 0.5 mM EDTA for 30 min on ice and washed, and the binding of fl-APC was determined in HBSS containing 1% BSA, 3 mM $CaCl_2$, 0.6 mM $MgCl_2$, 0.02% sodium azide, using methods described previously (6, 7). For some experiments, mAbs were purified from mouse ascites fluid on a protein G column (Pharmacia-LKB), following the supplier's protocol. In all cases, purified antibody was confirmed to retain EPCR-binding function by FACS analysis using E7 cells and detection of bound immunoglobulin with fluorescein-labeled goat anti-mouse IgG (Becton Dickinson). Binding was also confirmed by an ELISA method using sEPCR-HPC4 labeled with biotin on either primary amino groups or terminal sialic acid residues (essentially as described above for protein C) bound to avidin-coated microtiter plates. Immunoglobulin was detected with enzyme-conjugated anti-mouse IgG. All mAbs used in the current study were of the IgG1κ subclass.

Flow Cytometric Analysis of fl-APC-Blocking Anti-EPCR mAbs. The effect of the antibodies on binding of fl-APC, fl-cho-protein C, or biotin-PC to E7 cells, EA.hy926 cells, and HUVECs was studied by using a modification of previously described methods (6). Briefly, harvested cells were preincubated on ice for 30 min with an equal volume of 100 μg/ml purified antibody or conditioned hybridoma culture supernatant in HBSS/0.1% BSA/1 mM EDTA/0.02% sodium azide, pH 7.4. The cells were washed with ice-cold HBSS containing 0.1% BSA, and the cell pellet was resuspended in 100 μl of labeled protein (0–160 nM) in HBSS/0.1% BSA/3 mM $CaCl_2$/0.6 mM $MgCl_2$/0.02% sodium azide. Cells were incubated for 30 min on ice, 0.2 ml of the same buffer was added, and cell-bound fluorescence was determined by FACS with at least 5000 events counted per sample. Bound biotin-PC was detected by FACS analysis after incubation with 0.5 μg/ml streptavidin-phycoerythrin in the same buffer. The phycoerythrin fluorescence was detected on the FL2 channel with the voltage set to 500.

We analyzed 103 hybridomas and selected the best blockers, 1494 and 1496, for further analysis. JRK-1 was chosen to compare with the blockers because it did not block binding and was of the same subclass. The mean channel fluorescence intensities with CTM1009 (a TM marker) and JRK-1 (an EPCR marker) at optimal antibody concentration on EA.hy926 cells were within a factor of 2. APC binding to E7 cells is about 2–4 times greater than to EA.hy926 cells or HUVECs, which bind similar amounts. Greater than 90% of the APC binding is blocked by 1496 mAb on all cell types. Thus, the TM and EPCR surface densities appear to be similar.

Direct Binding Assay. sEPCR-HPC4 was labeled with biotin-LC-hydrazide by using the method described above for selective modification of sialic acid residues on protein C. Microtiter plates (96 wells) were coated overnight at 4°C with 50 μl of 10 μg/ml avidin in 0.1 M sodium carbonate, pH 9.6. The following steps were performed at room temperature. The wells were washed and then blocked for 1 hr with 0.1% (wt/vol) gelatin in 0.02 M Tris·HCl/0.1 M NaCl, pH 7.5. The wells were washed and incubated for 1 hr with 50 μl of the biotin-cho-sEPCR-HPC4 (70 nM). This concentration of labeled EPCR was chosen from preliminary titration curves of increasing APC concentration (0–1 μM) added to wells coated with labeled EPCR at increasing concentrations (0–2.38 μM). The wells were washed with the same buffer containing 3 mM $CaCl_2$, 0.6 mM $MgCl_2$. 0.05% Tween 20, and 0.02% sodium azide. mAbs were added (50 μl of 10 μg/ml), the mixture was incubated for 1 hr, and wells were washed with the same buffer. APC was added (50 μl of 0–1 μM), the mixture was incubated for 1 hr, and the wells were washed twice with the same buffer. The amidolytic activity of the bound APC was determined by addition of 50 μl of 0.4 mM Spectrozyme PCa, and the change in absorbance at 405 nm with time (mOD/min) was read on a Vmax microplate reader (Molecular Devices). All assays were done in at least triplicate wells on two different experiments. sEPCR-HPC4 labeled with biotin on primary amino groups gave essentially identical results (data not shown).

Clotting Assay. The effect of 1496 mAb on the ability of sEPCR-HPC4 to inhibit APC anticoagulant activity in a one-stage Xa clotting assay was performed as described (8) except that the EPCR was preincubated for 15 min with 200 μg/ml antibody at 37°C.

Protein C Activation Assays. Confluent EA.hy926 cells or HUVECs (48-well plates) were washed three times with HBSS containing 0.1% BSA and 0.02% sodium azide and were preincubated with 200 μl of buffer or 50 μg/ml mAb for 30 min at room temperature. The supernatant was aspirated, and cells were washed three times with HBSS/0.1% BSA/3 mM $CaCl_2$/

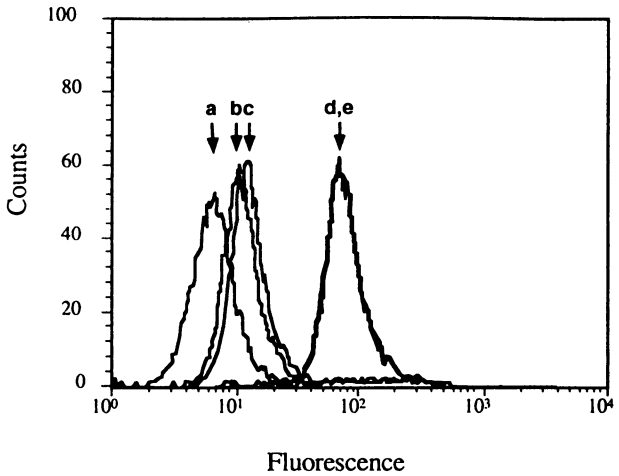

FIG. 1. Inhibition of fl-APC binding to EA.hy926 cells. EA.hy926 cells were preincubated with buffer or 50 μg/ml mAb (JRK-1, 1494, or 1496) in EDTA, followed by incubation with 80 nM fl-APC in buffer containing calcium and magnesium. The bound fluorescence intensity was determined by FACS analysis as indicated by arrows: a, control cells without fl-APC; b, 1494 mAb with fl-APC; c, 1496 mAb with fl-APC; d, JRK-1 with fl-APC; and e, fl-APC alone.

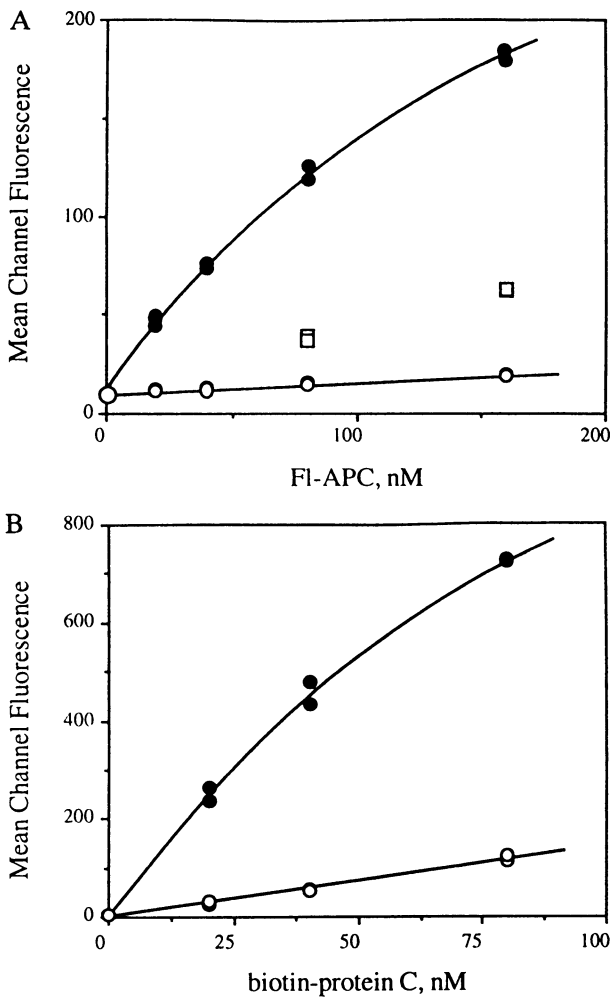

FIG. 2. Effect of anti-EPCR antibodies on fl-APC binding to EPCR. The binding of increasing concentrations of fl-APC (*A*) or biotin-PC (*B*) by EA.hy926 cells was determined by FACS analysis after preincubation in the presence (○) or absence (●) of 1496 mAb (50 μg/ml). Ligand binding in the presence of 1.5 μM unlabeled protein C was also determined (□). Biotin-PC binding was detected by incubation with streptavidin-phycoerythrin.

0.6 mM $MgCl_2$/0.02% sodium azide. Protein C (0.2 μM) or Gla-domainless protein C (1 μM) was added and the reaction was initiated by addition of thrombin (10 nM) in a total volume of 200 μl in the same buffer. At defined times, the reactions were stopped by addition of 150 μl of supernatant to 10 μl of antithrombin III (1.66 mg/ml) and heparin (1.4 units/ml), and the amidolytic activity of APC was determined by using 0.2 mM Spectrozyme PCa substrate and the Vmax microplate reader. For K_m and K_d determinations, the activation assays were done with either increasing protein C concentrations (0–2 μM) and 5 nM thrombin or increasing thrombin concentrations (0–16 nM) and 0.2 μM protein C in a total volume of 200 μl. All assays were done in at least triplicate wells, and less than 10% of the protein C substrate was activated as determined by reference to a standard curve of fresh fully activated protein C versus mOD/min. All kinetic constants were determined by analysis of the data using ENZFITTER (R. J. Leatherbarrow, Elsevier Biosoft).

RESULTS

mAbs were prepared against recombinant soluble EPCR and screened for the ability to block binding of both APC and protein C. Two antibodies, 1494 and 1496, blocked the binding of 80 nM fl-APC to transformed endothelial cells almost completely, whereas the JRK-1 anti-EPCR antibody did not inhibit (Fig. 1). All three antibodies bound essentially equivalently to both EA.hy926 cells and E7 cells (data not shown). The inhibition of ligand binding was observed over a range of fl-APC concentrations on all EPCR-expressing cell lines tested, including E7, EA.hy926, and HUVEC (Fig. 2*A* and Table 1). Protein C binding to EPCR was inhibited in a similar fashion, and inhibition was independent of the manner in which the protein C was labeled with the fluorophore (Fig. 2*B* and Table 1). Identical results were obtained in a purified system in which biotinylated EPCR bound to avidin-coated wells could support APC binding, and this binding was inhibited by 1496 mAb (Table 1). The data shown in Table 1 at a single ligand concentration were taken from saturation binding curves and are representative of the results at each concentration. Essentially identical results were obtained with purified 1494 mAb on E7 and EA.hy926 cells and with both blocking antibodies from hybridoma culture supernatants (data not shown). The JRK-1 antibody, either purified or from conditioned hybridoma culture supernatants, was consistently unable to affect fl-APC binding (>95% of the maximum mean cell fluorescence) to all cell lines tested. Thus, these 1494 and 1496 mAbs blocked protein C and APC binding to EPCR on cell surfaces and in a purified system, but the class-similar JRK-1 mAb failed to block binding.

In a previous study, we showed that soluble EPCR effectively inhibited the ability of APC to inactivate its natural substrate, factor Va, but did not alter the APC interaction with proteinase inhibitors (8). If these inhibitory antibodies bind to the ligand-binding site of EPCR, they should be able to reverse this EPCR-induced modulation of APC activity. As shown in Fig. 3, sEPCR-HPC4 again effectively decreased APC anticoagulant activity, and this was reversed by prior incubation of EPCR with the blocking 1496 mAb.

The availability of blocking antibodies allowed us to investigate the potential roles of EPCR in protein C activation. EA.hy926 cells are a hybrid cell line of human endothelium and lung carcinoma cells (17) that express both EPCR and TM as

Table 1. Inhibition of APC and protein C binding to cellular and purified EPCR

			EA.hy926 cells*							
	E7 cells* fl-APC		fl-APC		fl-cho-PC		HUVEC* fl-APC		Plate assay† APC	
Inhibitor	MCF	%	MCF	%	MCF	%	MCF	%	mOD/min	%
Buffer	113.24	100	39.65	100	50.85	100	38.89	100	1.594	100
1496 mAb	5.41	4.8	2.87	7.2	14.61	28.7	2.32	8.0	0.335	21.0
Protein C	27.73	24.5	9.44	23.8	ND		8.00	20.6	ND	

ND, not determined.

*The effect of 50 μg/ml 1496 mAb or 1.5 μM protein C on the binding of 80 nM fl-APC or fl-cho-protein C (fl-cho-PC) to E7 cells, EA.hy926 cells, or HUVEC was determined by FACS analysis. The mean cell fluorescence (MCF) and percent of maximum binding (%) are shown. The data shown are the average of duplicate samples with the background MCF subtracted.

†The binding of APC to immobilized biotin-cho-sEPCR-HPC4 was measured in a plate assay as described in the text, and bound APC amidolytic activity was determined. The data shown are corrected for background and are the average of quadruplicate wells at 0.25 μM APC added.

judged by binding of the appropriate mAbs and subsequent FACS analysis (data not shown). Preincubation with the blocking anti-EPCR antibodies reduced the rate of protein C activation to 20–33% of that of the control cells, but they had no effect on the ability of the cells to activate Gla-domainless protein C (Fig. 4*A*). As expected, protein C activation was not affected by unrelated antibodies or the non-blocking JRK-1 antibody. CTM1009, an antibody to TM that blocks thrombin binding, reduced activation to 8% of the control. Essentially identical results were obtained with HUVECs (Fig. 4*B*) with the consistent conclusion that EPCR alters protein C activation rates in a manner dependent on the presence of the protein C Gla domain.

Since the antibodies block APC/protein C binding to EPCR and inhibit the rate of protein C activation, it was of interest to determine whether this was an effect on protein C as a substrate for the thrombin–TM complex, or whether thrombin binding to TM was altered in some fashion. Analysis of the substrate dependence for thrombin-catalyzed protein C activation on EA.hy926 cells demonstrated a 3-fold increase in apparent K_m from 0.5 ± 0.1 μM in the absence of 1494 mAb to 1.6 ± 0.4 μM in the presence of the antibody (Fig. 5). There was also a small decrease in k_{cat} observed from 79.7 ± 7.7 sec^{-1} to 46.5 ± 7.1 sec^{-1}. In contrast, identical treatment of the cells with 1496 mAb had no effect on the ability of thrombin to bind to TM, as judged by essentially equivalent half-maximal activation rates obtained as a function of thrombin concentration, with apparent K_d = 1.42 ± 0.32 nM and 1.49 ± 0.20 nM in the absence and presence of antibody, respectively.

DISCUSSION

These studies illustrate that EPCR plays a major role in protein C activation. The inhibitory mAbs to EPCR decreased the rate of protein C activation without influencing activation of Gla-domainless protein C. A model illustrating our current concepts of EPCR involvement in protein C activation and function is shown in Fig. 6. It would appear likely that EPCR can enhance protein C activation by the thrombin-TM complex only when the two receptors are in close proximity. Therefore, since the anti-EPCR mAbs would have no effect on thrombin–TM activation by complexes devoid of EPCR, the 70–80% reduction in protein C activation rate observed when endothelium is treated with blocking antibodies constitutes the minimum impact that EPCR has on protein C activation.

Gla domain involvement in the binding is proposed in the model, on the basis of the observation that protein C, but not Gla-domainless protein C, binds to EPCR (6). A functional role for this interaction can be inferred by comparison of the activation properties of protein C and the Gla-domainless derivative. Protein C is activated much more rapidly than Gla-domainless protein C over the surface of endothelium, but in solution, activation of either substrate by the thrombin–TM complex proceeds at the same rate (9). In conjunction with the current data, it is likely that the Gla domain-dependent acceleration of protein C activation observed over endothelium is mediated by interaction of the Gla domain of protein C with EPCR. Consistent with this hypothesis, the binding affinities of soluble EPCR and cell-surface EPCR for APC are virtually identical, and binding in solution also requires the presence of the Gla domain (7). Gla domain-specific interaction with endothelial cell receptors is not unique to this system, since Gla domain participation in factor IX binding to the

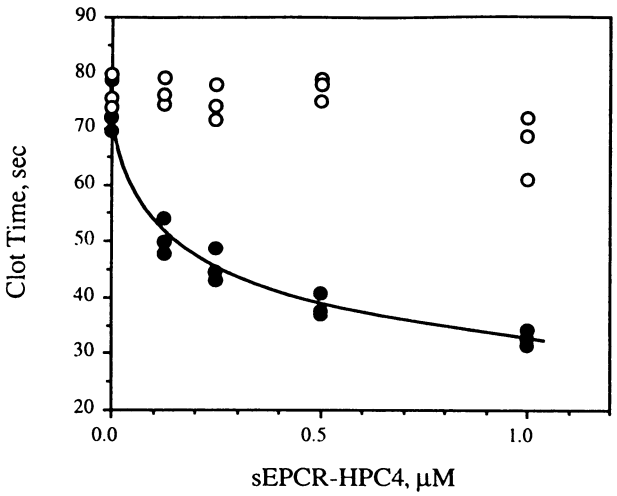

FIG. 3. Blocking antibody reverses the inhibition of APC anticoagulant activity by EPCR. The effect of soluble EPCR on the ability of APC to prolong the clotting time in a factor Xa one-stage clotting assay was determined as described in the text. sEPCR-HPC4 at the indicated concentrations was preincubated with buffer (●) or 200 μg/ml 1496 mAb (○) for 15 min at 37°C. APC was added to this mixture, followed by factor Xa and phospholipid, and clotting was initiated by the addition of human plasma. The final concentration of APC was 50 nM. The clotting time in the absence of added APC was 21.1 sec.

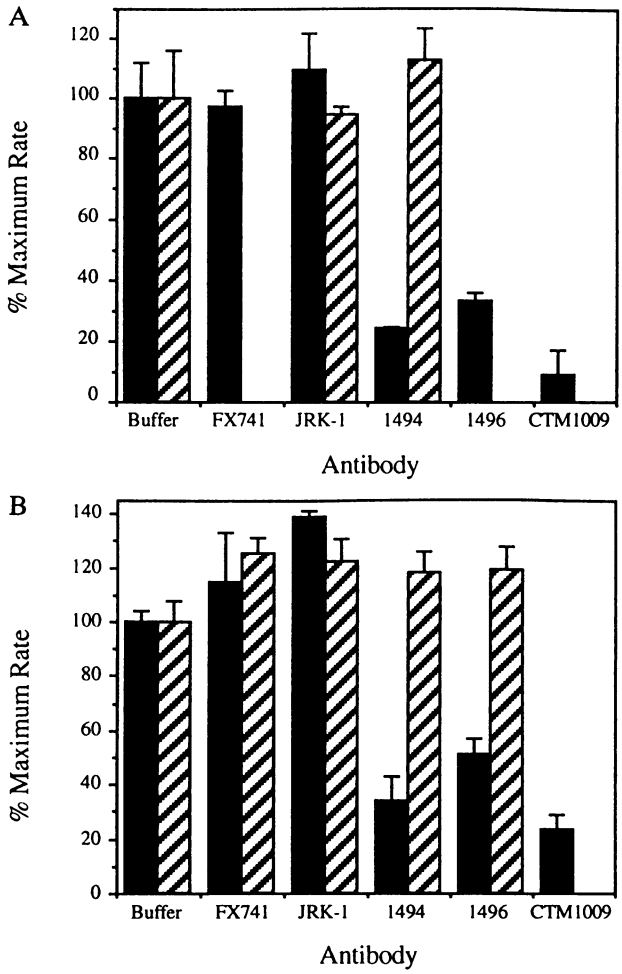

FIG. 4. Effect of anti-EPCR antibodies on protein C activation. Confluent EA.hy926 cells (*A*) or HUVECs (*B*) were preincubated at room temperature for 30 min with buffer or the indicated mAbs (50 μg/ml) and washed, and the rate of thrombin-dependent activation of protein C (solid bars) or recombinant Gla-domainless protein C (hatched bars) was determined on the cell monolayers. FX741, anti-human factor X; JRK-1, 1494, and 1496 are anti-EPCR antibodies; CTM1009, an anti-human TM antibody that blocks thrombin binding. Where Gla-domainless protein C activation data are not presented, no experiments were performed under those experimental conditions.

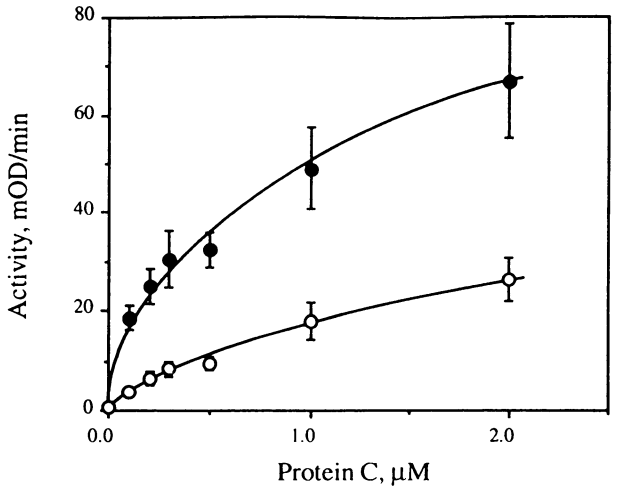

FIG. 5. Effect of 1494 mAb on the substrate dependence of protein C activation. The substrate dependence of protein C activation was determined on confluent EA.hy926 monolayers preincubated for 30 min with buffer (●) or 50 μg/ml 1494 mAb (○). Cells were washed, protein C was added at the indicated concentrations, and activation was initiated by thrombin (10 nM final). Supernatants were removed and added to a mixture of antithrombin III and heparin. The amidolytic activity of APC (mOD/min) was determined with Spectrozyme PCa.

endothelial cell factor IX receptor has been observed also (18). Thus, we tentatively propose that the EPCR binding to protein C is mediated at least in part by interactions with the Gla domain of protein C, although a Gla domain-dependent conformational change in protein C cannot be excluded.

From a physiological point of view, the interaction between EPCR and protein C appears to alleviate the requirement for protein C interaction with negatively charged phospholipid membranes. Likewise, thrombin, which lacks a Gla domain, interacts directly with TM through protein–protein interactions, and hence the assembly of the thrombin–TM complex does not have a requirement for negatively charged phospholipids. Negatively charged membranes can participate in all stages of the coagulation cascade, and hence the EPCR interaction with protein C and the interaction of thrombin with TM provide mechanisms for protein C activation without requiring a surface that could be used by other coagulation complexes. This is especially advantageous for the endothelium, which must maintain a nonthrombogenic surface.

The involvement of both EPCR and TM in protein C activation provides interesting possibilities in regulating the protein C pathway. Both receptors can be down-regulated by tumor necrosis factor α in endothelial cell culture. Selective down-regulation of EPCR could allow for endothelium to express TM that would activate protein C rather slowly but could still participate in thrombin clearance from the circulation and its inhibition by antithrombin or protein C inhibitor (19). Studies on the regulation and tissue distribution of EPCR should allow us to test this possibility.

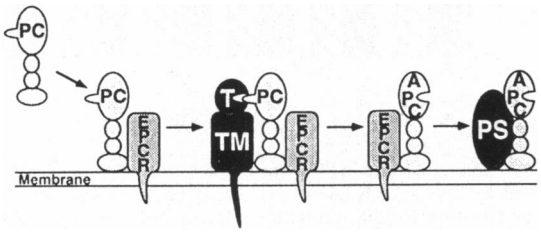

FIG. 6. Schematic summary of the role of EPCR in the protein C anticoagulant pathway. Plasma protein C binds to the endothelial cell protein C receptor, which then presents protein C to the thrombin–TM complex for activation. The activated protein C–EPCR complex either seeks alternative substrates or dissociates to initiate the anticoagulant pathway. PC, protein C; T, thrombin; PS, protein S.

EPCR promotes protein C activation, but it blocks the anticoagulant activity of APC (8). This inhibition of anticoagulant activity is not due to masking the active site of APC, since EPCR does not block chromogenic substrate hydrolysis or reactivity with two of the major plasma proteinase inhibitors of APC, α_1-antitrypsin and protein C inhibitor (8). We favor the concept that EPCR promotes protein C activation, and then the EPCR/APC complex has an altered enzyme specificity. This may be similar to the thrombin–TM complex in that thrombin interaction with TM blocks platelet activation and fibrinogen clotting but promotes activation of protein C. In this case, masking the factor Va recognition site may promote the activity toward other, yet-to-be identified, substrates. For APC to function as an anticoagulant on the endothelial cell surface, APC would apparently have to dissociate from EPCR and interact with protein S or other as-yet-unidentified receptors or binding sites (Fig. 6).

While these data provide new insights into the functions of EPCR, several questions remain unanswered. These include the nature of the interactions that govern the assembly of the protein C activation complex and the fate of the APC–EPCR complex. Further studies will be required to determine how the complexes assemble on the endothelial cell surface.

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